

Ms. A' COMPOSITIONS AND METHODS FOR ACTIVE VACCINATIONBACKGROUND OF THE INVENTION

This application relates to an active vaccine approach to the treatment of cancer and other diseases. The approach is applicable to a number of cancers and diseases, although a preferred embodiment provides an active vaccine for treatment of B cell Non-Hodgkin's Lymphoma (NHL).

NHL is characterized by a clonal proliferation of malignant B cells. The treatment of NHL across a broad spectrum of patients remains a challenge, although numerous therapeutic approaches have been proposed and tried.

The most common therapeutic approach being used today is chemotherapy. While chemotherapy is effective for some period of time in most patients, a significant percentage of patients are not cured and experience a relapse.

Treatments have been proposed based on anti-idiotypic therapy. In anti-idiotypic therapy, a cell surface molecule which is expressed by malignant cells but not by normal cells is used to create patient-specific antibodies which are then administered to the patient. See, Miller, et al., *New Engl. J. Med.* 306: 517-522 (1982). Autologous patient-derived idiotypic proteins have also been conjugated with keyhole limpet hemocyanin to produce a vaccine which has demonstrated efficacy and can elicit B and T cell immune responses. Kwak et al., *New Engl. J. Med.* 327: 1209-1215 (1992). Hybridoma-derived idiotypic was co-cultured with patient-derived dendritic cells which acted as antigen presenters upon re-infusion into the patient and showed clinical efficacy. Hsu et al., *Nature Medicine* 2: 52-58 (1996). Idiotypic vaccines made in lipid-based carriers are disclosed in International Patent Publication WO98/14170.

Treatments have also been proposed using antibodies directed to CD20, a transmembrane protein that is expressed by both normal and malignant B-cells during parts of the B cell development cycle. Using single-dose infusions with anti-CD20 monoclonal antibodies, partial or minor tumor regressions were observed in 6 of 15 patients in a Phase I clinical study. Maloney et al., *Blood* 84: 2457-2466 (1994). In Phase II studies, 17 of 37 patients showed complete or partial remissions. In December 1997, the FDA approved the

first antibody-based therapy for NHL. Rituximab (Ritvaxan, IDEC/Genentech) is a chimeric human/murine antibody approved for the treatment of patients with relapsed or refractory low-grade or follicular CD20⁺ B cell NHL. Maloney et al., *Blood* 90: 2188-2195 (1997).

Combinations of chemotherapy and anti-CD20 therapy have been reported as having better therapeutic efficacy, with 11 of 11 patients showing complete or partial remission. Czuczman et al., Abstract 53, *Ann. Oncol.* 7, Supp. 1: 56 (1996).

While therapeutic regimens using anti-CD20 concepts are potentially effective, all of these therapies have the drawback of being passive therapies, i.e., they do not directly involve the immune system of the patient. Thus, these therapies may require the continued administration of the therapeutic agent for efficacy and do not provide any long-term protection against recurrence. In addition, the passive therapy is monoclonal in nature, therefore escape is possible. It would therefore be desirable to have an active therapy, that is a therapeutic agent which when administered to the patient stimulates an immune response against CD20 found in B-cells.

It is an object of the present invention to provide such a therapy. It is a further object of the invention to provide an active polyclonal therapy that is difficult to evade.

SUMMARY OF THE INVENTION

In accordance with the present invention, NHL is treated, not by administration of an anti-CD20 monoclonal antibody, but by the administration of CD20 itself, or an immunogenic fragment of the extracellular portion thereof, coupled to or administered with an antigenic carrier moiety such as keyhole limpet hemocyanin (KLH). This results in the stimulation of the production of polyclonal antibodies against CD20 (or an immunogenic fragment thereof) which has the affect of reducing the number of B-cells, including malignant B-cells. Thus, the invention provides an active vaccine. The same approach can be used for therapeutics for other diseases and conditions in which target cells possess a transmembrane protein, and is particularly applicable to those diseases and conditions for which administration of antibodies to transmembrane proteins or peptides (i.e., passive therapy) have been shown to provide therapeutic benefits, and especially in the

situations where the target is also capable of transducing or receiving a signal important for cell growth or function. This would include, for example, Her2/neu, VEGF receptor, epidermal growth factor receptor, the CD19 molecule, interleukin-2-receptor, interleukin-4-receptor, and the P-glycoprotein, also known as the multidrug-resistance protein.

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BRIEF DESCRIPTION OF THE FIGURES

Figs. 1A and B show ELISA results for formation of antibodies to human and mouse CD20 in vaccinated mice;

Figs. 2A and B shows results for binding of control B1 antibodies or antibodies in plasma from a mouse treated with human CD20-KLH conjugate with Raji B NHL cells;

Fig. 3 shows CP19⁺ B cell levels in mice treated with human or mouse CD20-KLH conjugate;

Fig. 4 shows the domain structure of human Her2;

Fig. 5 shows the domain structure of human EGFR;

Figs. 6A-D shows the cross-reactivity of antibodies generated in response to human or mouse CD20 fragments;

Figs. 7A-D show the importance of carrier protein and adjuvant in generating an immune response;

Figs. 8A-D shows the immune response generated using different adjuvants; and

Figs. 9A-I shows CP19⁺ B cell levels in mice treated with human or mouse CD20-KLH conjugate.

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DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an active vaccine therapy which can be used in the treatment of a variety of cancers and related conditions in which it is desirable to bring about the death of a target group of cells. Conventionally, immunotherapies targeting cells have sought to obtain a cellular immune response (T-cells that recognize the target cells), since a humoral immune response (antibodies that recognize the target cells) alone is not

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deemed sufficient to achieve the desired result of cell death. The present invention departs from this conventional wisdom, and effectively utilizes a humoral immune response against the target cells to provide therapeutic benefit. The targets for therapy include cell surface proteins that when bound by a ligand signal to the cell. The vaccine induced antibody response will mimic ligand binding and cause similar signaling events which can imitate the process of programmed cell death (apoptosis) or halt the cell from growing or change the cancer cell's sensitivity to chemotherapy.

By way of example, the invention is suitably employed in the treatment of NHL and other B cell diseases such as chronic lymphocytic leukemia, auto-immune disorders and B-cell regulatory disorders. In accordance with this embodiment of the invention, a peptide antigen is prepared which contains at least an immunogenic portion of the extracellular domain of CD20 coupled to or administered with an antigenic carrier protein. The CD20 component of the peptide antigen may be syngeneic or it may be xenogeneic. Thus, for example, human patients may be treated with a peptide vaccine containing a human or a mouse CD20-fragment. There is evidence that strong immune responses can be elicited against xenogeneic proteins. Naftzger et al., *Proc. Natl. Acad. Sci. (USA)* 93: 14809-14814 (1996); International Patent Application PCT/US97/22669, filed December 10 1997, incorporated herein by reference. A suitable fragment is the 44 amino acid peptide spanning amino acids 136 to 179 of the sequence of mouse or human CD20. (Seq. ID Nos. 1 and 2) Other immunogenic fragments derived from the extracellular domain of CD20, or the entire CD20 molecule may also be used. Seq. ID. Nos. 3 and 4 shows the nucleic acid and amino acid sequences, respectively, of exon VI (the extracellular domain) of human CD20 as reported by Tedder et al., *J. Immunol.* 142: 2560-2568 (1989).

As used in the specification and claims hereof, an "immunogenic fragment" is a molecule which includes at least a portion of the extracellular domain of a transmembrane protein to direct and immunological response to that transmembrane protein when the immunogenic fragment is coupled to or administered with an antigenic carrier protein effective to break tolerance and administered with an adjuvant. It is not required that the immunogenic fragment alone be effective to stimulate an immune response, although such stimulation would not take a given fragment outside the scope of the present invention.

A preferred antigenic carrier protein is keyhole limpet hemocyanin which can be coupled to peptides using techniques described in Pierce Catalog Protocol. Other antigenic carrier proteins which can be used to break tolerance might be used in the invention include immunoglobulins, tuberculin, tetanus toxin and others well known in the art.

5 The peptide antigen containing the CD20 component and the antigenic carrier protein is formulated with a pharmaceutically acceptable adjuvant in a liquid carrier and administered to a patient suffering from NHL or another B cell disease. The composition will generally be administered by injection, for example, intramuscular, subcutaneous or intradermal injection, but might also be administered by way of a DNA vaccine (See US Patent No. 5,580,859, incorporated herein by reference) or a viral vaccine, or after mixing with antigen presenting cells (APC's) such as dendritic cells, *ex vivo*. Alternatively, the antigen may be administered without adjuvant by injection into a host prepared by prior or simultaneous injection of an immune adjuvant. Specific amounts to be administered to a patient can be determined by monitoring the titer of anti-CD20 antibodies developed by the patient, or by an average group of patients using well-known technology.

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20 When a peptide of the extracellular domain of human or mouse CD20 is coupled to KLH and administered with an adjuvant to mice, antibodies which react with CD20 are found in plasma. (Figs. 1A and B) These antibodies bind to Raji cells, a human lymphoma cell line, indicating the ability to bind to a cell expressing CD20. (Figs. 2A and B). Moreover, the number of CD19⁺ B cells present in mice injected with either of the two CD20-KLH conjugates declines substantially (~30% decrease relative to controls). (Figs. 3 and 9). The assay used to quantitate B cell depletion detects CD19 which is also expressed on immature B cells that are CD20⁻. Thus, the 30% depletion actually underestimates the efficacy of the vaccine against CD20⁺ B cells. .

25 Antibodies generated in mice after vaccination with human or mouse-derived CD20 fragments are specific for the peptides used, yet are capable of inducing immunity to the corresponding peptide from other species (Figs. 6A-D). Studies showed that in most instances the peptide, carrier protein and adjuvant are all needed for optimal response, although some responses were detected using less than all of the components. (Figs. 7A-D).

Several different adjuvants were also tested, and QS21 was found to be the most effective of those tested. (Fig. 8A-D).

While not intending to be bound by any particular mechanism, it is believed that the vaccines of the present invention are effective via at least two pathways. First, the generation of a humoral immune response to CD20 is effective to some extent to reduce the numbers of B cells bearing CD20 antigen in a manner consistent with normal immunological response to a target antigen. In addition, however, because CD20 has a signaling function, the binding of antibody to the CD20 moiety activates this signaling function to trigger apoptotic cell death. Such stimulation of apoptosis has been demonstrated to occur *in vitro* following passive treatments with a chimeric anti-CD20 antibody. Maloney et al., *Blood* 88 (Supp. 1): 637a (1996).

It is also possible that T cell mediated effector mechanisms are involved in the immune response. As evidence of this, we illustrate in Table 1 the mouse and human peptide sequences capable of binding to the corresponding mouse and human histocompatibility antigens. This information was derived from a search of the NIH Bioinformatics and Molecular Analysis Section HLA Binding Predictions database using the mouse and human CD20 amino acid sequences. (Parker et al., *J. Immunol.* 152: 163 (1994)).

While the method of the invention is illustrated here using CD20 or CD20-derived peptides as the antigen to target B cells, the invention is not limited to this embodiment. Rather, the inventions encompasses the use of vaccine compositions comprising an immunogenic portions of the extracellular domain of transmembrane protein or peptide, particularly a transmembrane protein or peptide having signaling function, coupled to or administered with an antigenic protein and/or adjuvant to break tolerance.

A non-limiting example of another transmembrane protein which can be used in whole or in part in the method of the invention is Her-2/neu. The Her-2/neu oncogene is a receptor-like tyrosine kinase that is expressed on the cell surface of a significant portion of solid tumors. It has been shown that patients with early stage breast cancer have a high titer of antibodies to Her-2/neu. Disis et al., *J. Clin. Oncol.* 15: 3363-3367 (19967). The amino acid sequence and domain structure of human Her-2/neu are shown in Seq. OD. No. 5 and Fig. 4, and isolation and expression of the extracellular domain has been disclosed.

International Patent Publication No. WO 90/14357, which is incorporated herein by reference. There is clinical data showing efficacy of monoclonal antibodies against Her-2-neu in the treatment of patients with Her-2/neu⁺ tumors, and potential synergism with chemotherapy. Thus, in accordance with the present invention, a vaccine composition comprising at least an immunogenic portion of the extracellular domain of Her-2-neu (amino acids 22 to 652) coupled to or administered with an antigenic protein or peptide such a KLH can be used as a vaccine to provide the same therapeutic benefits using an active as opposed to a passive approach.

A further non-limiting example of a transmembrane protein which can be used in whole or in part in the method of the invention is epidermal growth factor receptor (EGFR). The amino acid sequence and domain structure of human EGFR are shown in Seq. ID. No. 6 and Fig. 5. There is significant data showing that antibodies to EGFR can have anti-tumor activity in breast and prostate cancer, as well as several head and neck tumors. Prewett et al., *J. Immunother. Emphasis Tumor Humoral* 19: 419-27 (1996). The mechanism by which antibody therapy against EGFR may be efficacious can be through the ability to down-regulate vascular endothelial growth factor production by tumor cells and thereby decrease angiogenesis. Petit et al., *Am. J. Pathol.* 151: 1523-30 (1997). In accordance with the present invention, a vaccine composition comprising at least an immunogenic portion of the extracellular domain of EGFR (amino acids 25 to 645) coupled to or administered with an antigenic protein or peptide such a KLH can be used as a vaccine to provide the same therapeutic benefits using an active as opposed to a passive approach. Preferred immunogenic peptides would be selected from regions not deleted in the various types of truncated EGFR mutants associated with some cancers.

A further non-limiting example of a transmembrane protein which can be used in whole or in part in the method of the invention is VEGF receptor. There are significant data showing that antibodies to VEGF receptor can inhibit angiogenesis and thereby halt tumor progression. In accordance with the present invention, a vaccine composition comprising at least an immunogenic portion of the extracellular domain of VEGF receptor coupled to or administered with an antigenic protein or peptide such a KLH can be used as a

vaccine to provide the same therapeutic benefits using an active as opposed to a passive approach.

Still a further non-limiting example of a transmembrane protein which can be used in whole or in part in the method of the invention is the IL-2 receptor. The IL-2 receptor is expressed on most T-cells malignancies, and there is a data showing that antibodies to the IL-2 receptor can be used in the treatment of T-cell malignancies and autoimmune disorders. In the present invention, a composition is made comprising at least an immunogenic portion of the extracellular domain of the IL-2 receptor (e.g., P55 or P75), coupled to or administered with an antigenic carrier protein or peptide such as KLH. and used as a vaccine.

The vaccine compositions of invention can be used alone or in combination (concurrently or sequentially) with drugs or chemotherapy agents that provide therapeutic benefit for the condition being treated. In the case of NHL, suitable chemotherapy agents which can be used in combination with the CD20 based vaccine include alkylating agents, anthrocyclines, cis-platinum, fludarabine, corticosteroids and vinca alkaloids. These same chemotherapy agents which might be used in combination with other vaccine compositions for other forms of cancer.

EXAMPLE 1

44 amino acid fragments of the extracellular domains of humans and murine CD20 (amino acids 136-179, Seq. ID Nos. 1 and 2) were synthesized using a solid-phase FMOC peptide synthesizer and coupled to KLH using the methodology described in the Pierce Catalog Protocol. The peptide coupled to KLH was then prepared for injection by formulation with QS-21 adjuvant. Balb/c mice were injected according to one of the following protocols on days 1, 8, 15, 22 and 50 of the experiment:

- A. Murine CD20 fragment-KLH with QS-21 adjuvant
- B. Human CD20 fragment-KLH with QS-21 adjuvant
- C. KLH with QS-21 adjuvant
- D. QS-21 adjuvant

- E. P190 (irrelevant protein) coupled to KLH with QS-21 adjuvant
- F. B3A2 (irrelevant peptide) coupled to KLH with QS-21 adjuvant.

The animals were sacrificed on day 62 of the experiment.

5 Serum samples from the mice were diluted 1:200 and evaluated by BSA-blocked ELISA using goat-anti-mouse antibody conjugated to alkaline phosphatase for antibodies which bind to human CD20, mouse CD20 and KLH. As shown in Figs 1A and B, mice injected with human CD20 coupled to KLH (Fig. 1A) or mouse CD20 coupled to KLH (Fig. 1B) administration of xenogeneic antibody produced a significant polyclonal antibody response to both human and mouse CD20, while the response following administration of syngeneic antibody was principally limited to antibodies to the syngeneic form of CD20. Either xenogeneic or syngeneic peptide can therefore be used to generate an immune response.

To confirm the ability of the antibodies to bind to B cells, Raji cells (a form of human B-cell lymphoma that expresses CD20 on its surface) were blocked with human IgG, washed and then incubated for 30 minutes on ice with a 1:10 dilution of plasma from a mouse vaccinated with P-190-KLH control or huCD20-KLH. As a positive control, Raji cells were incubated with B1 antibody, or IgG2 as an isotypic negative control. After washing, the cells were incubated with goat-anti-mouse antibody, washed and fixed with 1% paraformaldehyde.

20 Flow cytometry analysis was performed in a Becton-Dickinson FACScan. The results are shown in Figs 2A and 3B, wherein the shaded data set are the experimental data set and the outlined data set is the negative controls. As is apparent, there is a strong binding of mouse antibodies and Raji cells, comparable to that observed with B1 antibody .

25 EXAMPLE 2

To assess the number of B cells present in vaccinated mice, an evaluation was made of cells expressing CD19, a standard phenotypic marker for B cells. Spleens were harvested from the animals vaccinated in Example 1 and put into a single-cell suspension. After counting the total number of cells, the cells were stained with FITC-labeled anti-mouse

30 CD19 and the samples were analyzed by flow cytometry with a FACScan. 10,000 events

were collected. The percentage of CD19 positive cells minus the control gate was multiplied by the total number of cells to determine the number of CD19 positive cells in mice treated with the mouse and human CD20 peptide conjugates, and the P190 irrelevant peptide conjugate control.

As shown in Fig. 3, the absolute number of CD19 positive cells was significantly reduced in mice treated with either of the CD20 peptide conjugates. The level of CD19 positive cells is a reflection of the number of CD20 positive B cells, and the number of immature CD19⁺, CD20⁻ B cells in the samples. The absolute number of CD19⁺ B cells actually underestimates the therapeutic efficacy of the treatment for elimination of CD20⁺ B cells, however, since CD19 is expressed on B cell progenitor cells before expression of CD20.

EXAMPLE 3

Mice were injected five times over two months with one of four treatment protocols as follows:

human CD20 (44 aa fragment)-KLH plus QS1

human CD20 (44 aa fragment)-KLH

human CD20 (44 aa fragment) plus QS21

KLH plus QS21

Blood was collected on week 9 for analysis by ELISA. Sera from the vaccinated mice were diluted 1:200 and incubated on BSA blocked plates coated with msCD20, huCD20, P190 or KLH. Secondary goat anti-mouse antibody conjugated to alkaline phosphatase was added, and the color change of p-nitrophenyl phosphate substrate was measured at 405 nm. The results are summarized in Figs. 7A-D. In most instances the peptide, carrier protein and adjuvant are all needed for optimal response, although some responses were detected using less than all of the components.

EXAMPLE 4

Mice were vaccinated according to the schedule of Example 3 using one of four treatment protocols: human CD20 (44 aa fragment)-KLH plus QS21 adjuvant, mouse

CD20 (44 amino acid fragment)-KLH plus QS21, P190 (irrelevant protein)-KLH + QS21 and KLH and QS21 alone. Mouse serum samples were evaluated by ELISA for the presence of antibodies reactive with msCD20, huCD20, P190 and KLH. The results are shown in Figs. 6A-D. Antibodies generated in mice after vaccination with human or mouse-derived CD20 fragments are specific for the peptides used, yet are capable of inducing immunity to the corresponding peptide from other species.

EXAMPLE 5

Mice were vaccinated five times over two months with huCD20 fragment-KLH conjugate with no adjuvant or in combination with one of three adjuvants: QS21, BCG or Alum. Serum samples from the vaccinated mice were tested by ELISA. The results are summarized in Figs. 8A-D. QS21 was found to be the most effective of those tested.

EXAMPLE 6

To confirm the observations of Example 2, nucleated spleen cells were recovered by centrifugation in a density gradient from mice vaccinated with a CD20-KLH conjugate (human or mouse) in the presence of QS21 adjuvant. 1×10^6 cells from each mouse were incubated with 2 μ g of rat anti-mouse CD19 FITC-labeled antibody or with isotope-matched FITC labeled rat antibody. Cells were washed, fixed and analyzed with a Becton Dickinson FACScaliber cytometer. Figs 9A-C, D-F and G-I show the results for three exemplary mice of each vaccination group. The decrease in the peak reflecting levels of CD19 positive spleen cells in each of the mice is apparent.

Table 1

Species	Peptide Sequence	HLA Molecule	Score (T _{1/2} of Dissociation of Molecule Containing this Subsequence)
human	NFIRaHTPYI	Kd	1600
human	FIRaHTPYI	Kd	48
human	FLKMcSLNFI	A_0201	21.2
mouse	HFLKmRRLEL	Kd	1600
mouse	IYDCePSNSS	Kd	60
mouse	LIQTSKPYV	A_0201	89.4
mouse	ELIQSKPYV	A_0201	28.7